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Morphological and Genetic Evidence that the Cyanobacterium *Lyngbya wollei* (Farlow ex Gomont) Speziale and Dyck Encompasses at Least Two Species▽†

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Dense blooms of the cyanobacterium *Lyngbya wollei* are increasingly responsible for declining water quality and habitat degradation in numerous springs, rivers, and reservoirs. This research represents the first molecular phylogenetic analysis of *L. wollei* in comparison with the traditional morphological characterization of this species. Specimens were collected from several springs in Florida and a reservoir in North Carolina. Segments of the small-subunit (SSU) rRNA and *nifH* genes were PCR amplified, cloned, and sequenced. The phylogenetic analysis of the SSU rRNA gene revealed sequences that fell into three distinct subclusters, each with >97% sequence similarity. These were designated operational taxonomic unit 1 (OTU1), OTU2, and OTU3. Similarly, the *nifH* sequences fell into three distinct subclusters named S1, S2, and S3. When either bulk samples or individual filaments were analyzed, we recovered OTU1 with S1, OTU2 with S2, and OTU3 with S3. The coherence between the three SSU rRNA gene and *nifH* subclusters was consistent with genetically distinct strains or species. Cells associated with subclusters OTU3 and S3 were significantly wider and longer than those associated with other subclusters. The combined molecular and morphological data indicate that the species commonly identified as *L. wollei* in the literature represents two or possibly more species. Springs containing OTU3 and S3 demonstrated lower ion concentrations than other collection sites. Geographical locations of *Lyngbya* subclusters did not correlate with residual dissolved inorganic nitrogen or phosphorus concentrations. This study emphasizes the need to complement traditional identification with molecular characterization to more definitively detect and characterize harmful cyanobacterial species or strains.

Lyngbya wollei is a filamentous, nonheterocystous cyanobacterium that is capable of nitrogen (N_2) fixation. The filaments are encased in a sheath, and the cells have been observed to have a width of 24 to 65 μm and a length of 2 to 12 μm (19). *L. wollei* is considered a harmful cyanobacterium, or cyanoHAB, because of its ability to produce neurotoxins and hepatotoxins (2, 17, 28) and its tendency to form dense mats that smother submerged aquatic vegetation (20, 21). Public concern in Florida has grown in recent years over the large number of springs, lakes, and rivers (19 in this study) that increasingly support massive benthic and/or floating *L. wollei* mats that adversely affect water quality. *L. wollei* is known to thrive in environments with a wide range of N concentrations (5, 23). This is due to its abilities to rapidly acquire combined N from the water column when it is abundant and to fix atmospheric N when combined N concentrations are low (6, 12). In Florida, *L. wollei* has become increasingly common in springs that have high flow rates, low salinity, and low combined N concentrations (23; http://nwis.waterdata.usgs.gov/fl/nwis/qwdata).

Because of the amount of environmental degradation and potential health problems associated with *Lyngbya* blooms, there is interest in managing their occurrence. An effective management strategy, however, may depend, in part, on knowing whether or not *L. wollei* comprises one or several species which may vary in their environmental requirements, physiological characteristics, and toxicity. The advent of molecular techniques has shown that morphologically defined species can, in fact, be multiple genetically distinct species which can have different habitat preferences (3). The specific sequences selected for analysis included regions of both the small-subunit (SSU) rRNA and *nifH* genes. SSU rRNA plays a crucial catalytic function in protein synthesis, and the corresponding gene has historically proven to be useful for the identification of cyanobacterial species (10, 24). Cyanobacterial SSU rRNA gene sequences collected from environmental samples are often classified into operational taxonomic units (OTUs), i.e., groups that are >97% similar (22, 27). The *nifH* gene encodes a crucial Fe protein subunit, dinitrogenase reductase, involved in N_2 fixation and has also been used extensively for the taxonomic characterization of cyanobacterial species (29).

In this study, *L. wollei* samples were collected from geographically dispersed environments and sequenced. Geographically dispersed sampling made it possible to assess the coherence between the SSU rRNA and *nifH* gene subclusters, as well as the distribution of specific strains or species showing >97% SSU rRNA gene sequence similarity (OTUs). At the same time, cell measurements on filaments collected at various sites were made to determine whether or not they fell into the size range ascribed to *L. wollei* (19). To determine if what is normally identified in sampling programs as *L. wollei* encom-

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TABLE 1. Collection sites for the *Lyngbya* sp. samples used in this study

Location	State	Latitude[a]	Longitude[a]
City Lake, High Point	North Carolina	35°59′43″N[b]	79°56′48″W[b]
Alexander Springs	Florida	29°04′50″N	81°34′30″W
Chassahowitzka Springs	Florida	28°42′54″N	82°34′35″W
Kings Bay	Florida	28°53′56″N	82°35′48″W
Fern Hammock Springs	Florida	29°11′00″N	81°42′29″W
Gainer Springs	Florida	30°25′35″N	85°32′53″W
Homosassa Springs	Florida	28°47′58″N	82°35′20″W
Ichetucknee Springs	Florida	29°58′47″N	82°45′31″W
Indian Springs	Florida	30°31′24″N	84°47′28″W
Juniper Springs	Florida	29°11′01″N	81°42′46″W
Rainbow Springs	Florida	29°11′01″N	81°42′46″W
Silver Glen Springs	Florida	29°14′43″N	81°38′37″W
Silver Springs	Florida	29°12′57″N	82°03′11″W
St. Johns River	Florida	29°35′40″N[b]	81°39′33″W[b]
Wakulla Spring	Florida	30°14′05″N	84°18′10″W
Washington Spring	Florida	30°27′12″N	85°31′52″W
Weeki Wachee Spring	Florida	28°31′00″N	82°34′25″W
Wekiwa Springs	Florida	28°42′43″N	81°27′36″W
Williford Spring	Florida	30°26′21″N	85°32′52″W
Wilson Spring	Florida	29°53′59″N	82°45′31″W
Withlacoochee River	Florida	29°2′46″N[b]	82°27′53″W[b]

[a] Latitudes and longitudes are from reference 16.
[b] Determined from Google Earth, 2007.

passed distinct strains or species, samples were taken from a number of springs, lakes, and rivers for both morphometric analysis and sequencing. The morphometric measurements were used to determine if the samples collected met the published criterion for this species and for comparison with the resulting phylogenetic analysis (19). Further, environmental data, including flow rates (14, 18), soluble-nutrient concentrations (http://nwis.waterdata.usgs.gov/fl/nwis/qwdata), and major ion concentrations (18, 25, 26), were collected to determine if any of the identified OTUs exhibited habitat preferences.

MATERIALS AND METHODS

Sample collection. *L. wollei* samples were collected from the sites listed in Table 1. Scientists involved with local water monitoring programs (Greenwater Laboratories, Palatka, FL; St. Johns River Water Management District, FL; and R. J. Stevenson) informed us of Florida springs that contained *Lyngbya* species, and these springs were chosen for sampling. Most sites were sampled one to three times, except Alexander Spring and Silver Glen Springs, which were sampled five and six times, respectively. *Lyngbya* was sampled at random from each spring. The darkest pigmented portions of the benthic mats (usually at a 1-m depth) were used for sample collection. Fresh *L. wollei* mat samples were immediately preserved for DNA analysis by placing an equal volume of *Lyngbya* filaments and buffer (10 mM Tris, 1 mM Na_2EDTA, pH 7.6) in a sterile capped plastic tube. The samples were quick-frozen on site and placed on ice during transport back to the University of North Carolina at Chapel Hill Institute of Marine Sciences, Morehead City. Thereafter, the samples were maintained at −20°C until DNA extraction was performed.

DNA extraction. DNA was extracted from whole *L. wollei* mats by placing ~100 mg (wet weight) in a tube containing glass beads. The samples were then alternated between an 80°C water bath for 1 h and at least 1 min in a bead beater (Biospec Products, Inc., OK) to lyse the cells. DNA was then purified with a DNeasy Plant Kit (Qiagen, Valencia, CA). A blank extraction control where reagents, but no cells, were extracted in the same manner as a normal sample was included during each DNA extraction to control for cross-contamination between samples. Single-filament extractions were performed as previously described (9).

PCR amplification. From each genomic DNA extraction, both a 324-bp segment of the *nifH* gene and a 374-bp segment of the SSU rRNA gene were PCR amplified with cyanobacterium-specific primers (10, 11). PCR amplicons were gel purified, cloned into the pCR 2.1-TOPO vector (Invitrogen, San Diego, CA), and sequenced at the University of North Carolina at Chapel Hill Automated DNA Sequencing Facility with a model 373A DNA sequencer (Applied Biosystems, Foster City, CA) by the *Taq* DyeDeoxy Terminator Cycle Sequencing method (Applied Biosystems, Foster City, CA).

Phylogenetic analysis. Sequences were aligned with the SeqLab program (GCG version 11.1; Accelrys Inc., San Diego, CA) and adjusted manually when individual nucleotides were obviously misaligned. Maximum-likelihood phylogenies were constructed with MrBayes (8, 13) by using the HKY-G model (Fig. 1a) and GTR+I+G (Fig. 2) for the SSU rRNA gene and the HKY model for *nifH*. These specific evolutionary models were selected as optimal after analysis of the alignments with the MrModelTest v. 2.2 program (distributed by J. A. A. Nylander). To simplify the graphical representation of the SSU rRNA gene and *nifH* phylogenies, the sequences that were 100% homologous or varied by relatively few base pairs were represented by a single sequence (Fig. 1; see also Table S1 in the supplemental material).

Morphometric analysis. The length and width of *L. wollei* cells from each sample were measured with a Nikon Eclipse E800 phase-contrast microscope at a magnification of ×200 and an ocular micrometer. A total of 1,392 measurements were taken (OTU1, 256; OTU2, 309; OTU3, 64; S1, 415; S2, 245; S3, 103) (Table 2).

Environmental parameters. To characterize the environments where *Lyngbya* was found, historical environmental data from 1990 to the present were assembled from previous studies. These data included ambient dissolved-nutrient and ion concentrations (4, 7, 15; http://nwis.waterdata.usgs.gov/fl/nwis/qwdata). Because the data collection frequencies varied significantly (26), it was decided that the only feasible way to conduct cross-environment comparisons was to use an average of the total data available at each site. To characterize the average ion composition of the various collecting sites, we collated the data from the studies of Whitford (25), Woodruff (26), and/or Slack and Rosenau (18) (Fig. 3 and Table 3). Each of these studies developed a classification scheme where the average salinity and ion content, including calcium, bicarbonate, magnesium, and sulfate concentrations, were ranked from low to high on a three-level scale. The "soft freshwater" and "hard freshwater" categories of Whitford (25) are based on calcium bicarbonate content. Their values fall into the lowest total-ion category established by Woodruff (26) and Slack and Rosenau (18). Because of this, we combined them into a single spring category called "calcium bicarbonate" (Table 3), which allowed a more accurate comparison between springs.

Soluble-nutrient values were determined for water samples obtained during *L. wollei* collections made at City Lake, NC, from summer 2001 and 2002 samplings and in the St. Johns River for three sampling events during 2004. The goal was to determine if there was a correlation between ambient soluble-nutrient levels and the occurrence of *Lyngbya*. The specific nutrients measured included NO_3^- plus NO_2^- [NO_x], NH_4^+, and PO_4^{3-}. Concentrations were determined by standard Lachat Quick-chem 8000 autoanalyzer (Lachat, Milwaukee, WI) protocols. The detection limits were 3.68 μg/liter ($N\text{-}NO_x$), 4.31 μg/liter ($N\text{-}NH_4^+$), and 0.74 μg/liter ($P\text{-}PO_4^{3-}$).

Statistical analysis. Statistical analysis consisted of a one-way analysis of variance with SPSS 11.0 software (SPSS Inc., Chicago, IL). A posteriori multiple comparison of means was achieved with the Bonferroni procedure with $\alpha = 0.05$.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study were submitted to GenBank and assigned accession numbers EF397755 to EF397766, EF397768, EF397770, EF397772 to EF39845, EF39847 to EF397908, EF422067, EF450886, EF450892, EF450894, EF450896, EF450900 to EF450903, EF450907, EF450911, EF450912, EF450919, EF450920, EF450922, EF450923, EF450926, EF450928, EF450932, EF450933, EF450937 to EF450939, EF450941, EF450946 to EF450968, EF450975, EF450978, and EF450980 to EF450996.

RESULTS

Phylogenetic analysis results. A phylogenetic analysis was carried out to determine if the various *L. wollei* SSU rRNA gene and *nifH* sequences segregated into distinct clusters indicative of strain or species level differences. The phylogenetic analyses showed that both the SSU rRNA gene and *nifH* sequences fell into three distinct clusters (Fig. 1). These group-

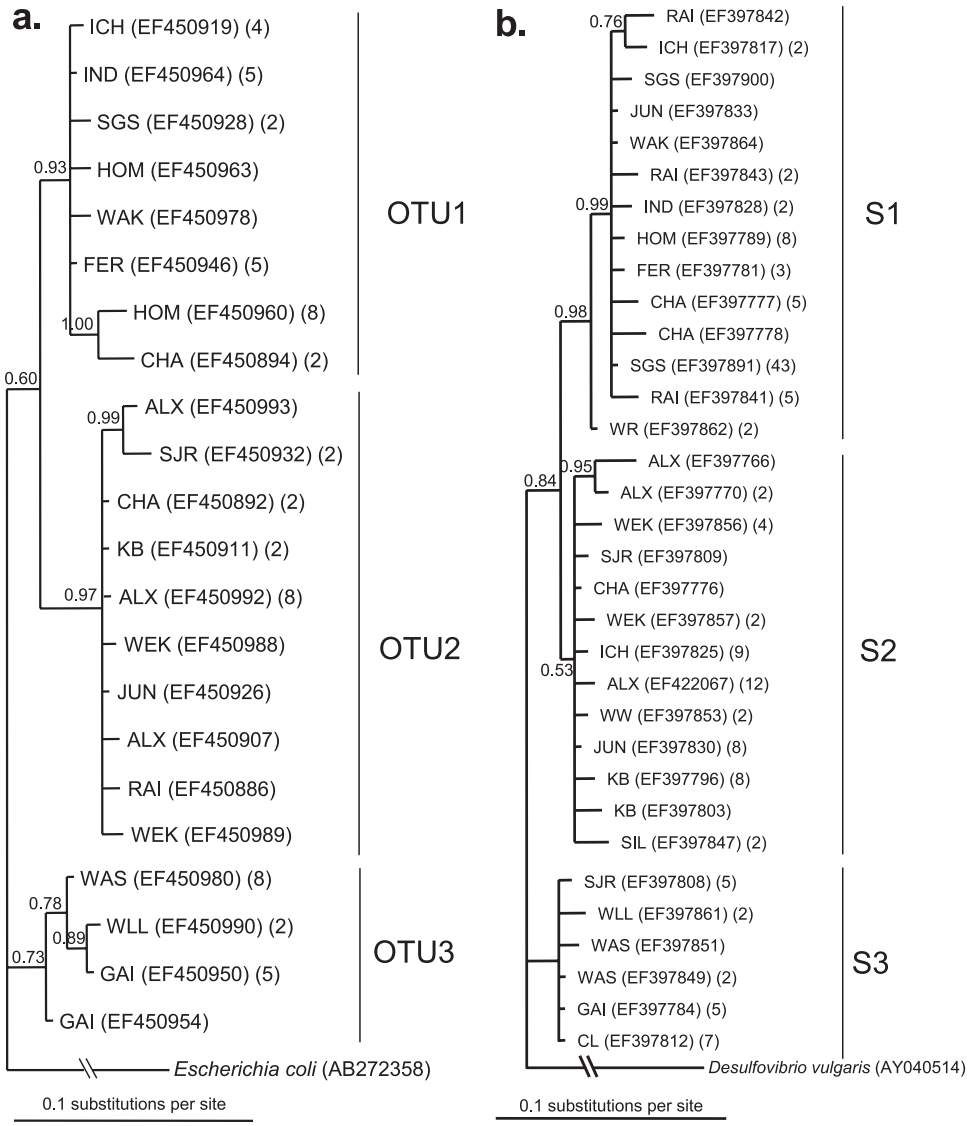

FIG. 1. Phylogenetic distribution of *L. wollei* partial 374-bp SSU rRNA gene (a) and 324-bp *nifH* (b) DNA sequences obtained in this study on the basis of Bayesian inference of phylogeny under HKY-G (a) and HKY (b) evolutionary models. Values at nodes are clade posterior probabilities. Values in parentheses are the numbers of replicate sequences with >97% base pair matches from the same site (see Table S1 in the supplemental material for the accession numbers of the sequences represented). Letter-and-number combinations in parentheses are NCBI database accession numbers. Abbreviations: CL, City Lake, High Point; ALX, Alexander Springs; CHA, Chassahowitzka Springs; KB, Kings Bay; FER, Fern Hammock Springs; GAI, Gainer Springs; HOM, Homosassa Springs; ICH, Ichetucknee Springs; IND, Indian Springs; JUN, Juniper Springs; RAI, Rainbow Springs; SGS, Silver Glen Springs; SIL, Silver Springs; SJR, St. Johns River; WAK, Wakulla Spring; WAS, Washington Spring; WW, Weeki Wachee Spring; WEK, Wekiwa Springs; WLL, Williford Spring; WLS, Wilson Spring; WR, Withlacoochee River.

ings were consistent whether the entire data set was used (data not shown) or a representative subset of the sequences was used (Fig. 1). These subclusters also consistently grouped together such that when SSU rRNA gene OTU1 was collected at a given location, *nifH* S1 was also recovered (Fig. 1). The same was true for the associations of OTU2 with S2 and OTU3 with S3. SSU rRNA gene OTU1 and OTU2 were strongly supported by posterior probabilities of >0.93 (Fig. 1a). In contrast, only the *nifH* S1 clade was strongly supported (posterior probability of 0.98; Fig. 1b). When the SSU rRNA gene and *nifH* sequences were obtained from a subset of single washed *L. wollei* filaments, the same associations of SSU rRNA gene OTU1 with *nifH* S1, OTU2 with S2, and OTU3 with S3 were observed (Table 4). These results were consistent with these sequences originating from distinct strains or species. Compared with other cyanobacterial SSU rRNA gene sequences, the *Lyngbya* OTUs identified from Florida and North Carolina in this study were distinct from those of other cyanobacteria, including other *Lyngbya* spp. (Fig. 2). The SSU rRNA gene phylogeny also showed that various *Lyngbya* species were dispersed throughout the tree and did not fall into a distinct genus-specific clade.

Morphometric analysis. To determine if the putative *L. wollei* identified within this study matched the taxonomic criteria

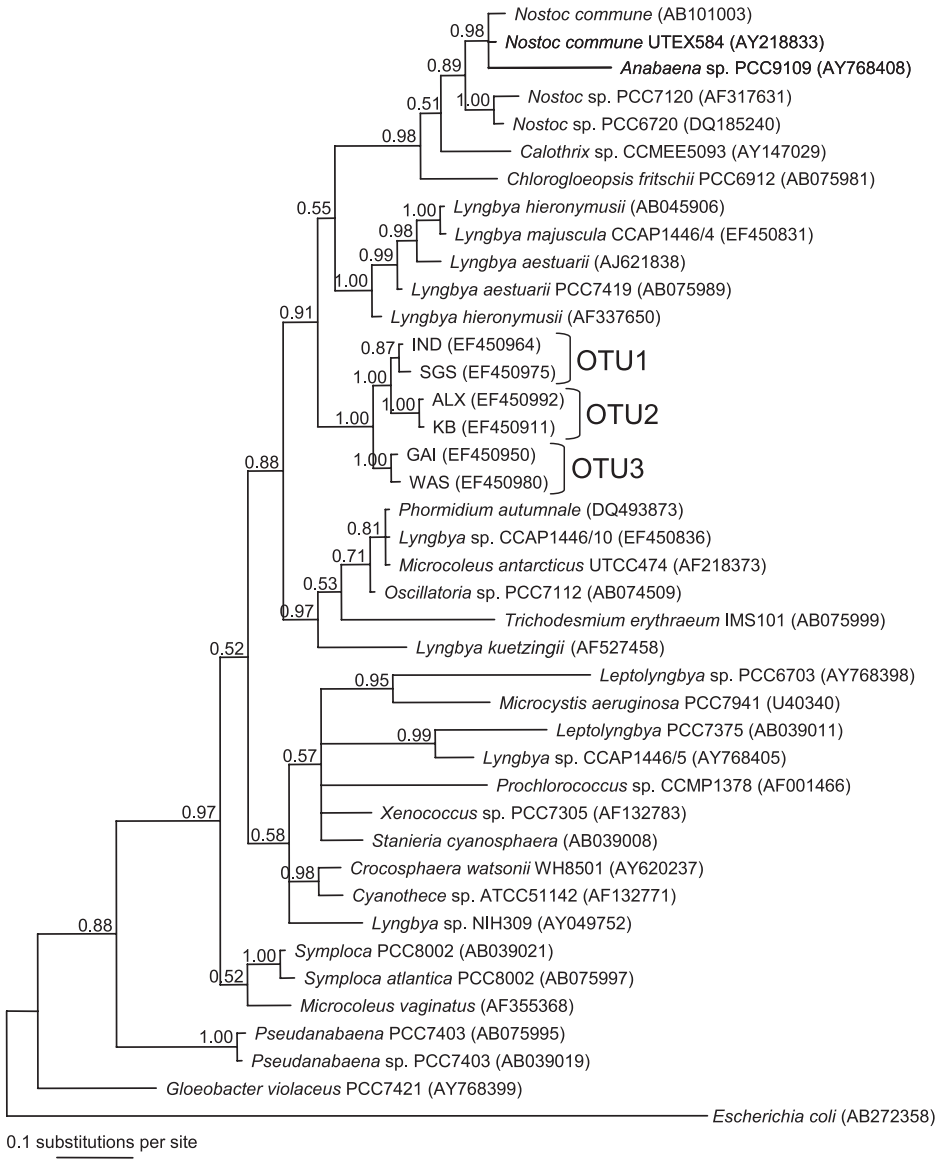

FIG. 2. Phylogenetic distribution of cyanobacteria and six representative *L. wollei* partial 374-bp SSU rRNA gene sequences obtained in this study on the basis of Bayesian inference of phylogeny under a GTR+I+G evolutionary model. Node values are clade posterior probabilities. Letter-and-number combinations in parentheses are NCBI database accession numbers.

TABLE 2. Cell measurements for *Lyngbya* sp. cells from each SSU rRNA gene (OTU) and *nifH* (S) sequence grouping

OTU or cluster	*n*	Width (μm)				Length (μm)			
		Max	Min	Avg	SE	Max	Min	Avg	SE
OTU1	256	56	23	41.4	0.33	9	2	5.1	0.18
OTU2	309	65	29	40.9	0.38	10	3	5.8	0.20
OTU3	64	72	44	56.5[a]	1.17	12	3	7.4[a]	0.47
S1	415	65	23	41.6	0.29	9	2	5.2	0.15
S2	245	65	30	41.3	0.39	10	2.5	5.7	0.27
S3	103	72	29	50.5[a]	1.43	12	3	7.0[a]	0.33

[a] Significantly different ($P < 0.05$) from other averages in the same gene group (SSU rRNA gene or *nifH*) and column.

of Speziale and Dyck (19), the widths and lengths of individual cells from each sample were measured by light microscopy. The widths of cells measured in the Speziale and Dyck (19) study ranged from 24 to 65 μm, and the lengths ranged from 2 to 12 μm.

Once the length and width data from each sample were recorded, these data were also segregated on the basis of their SSU rRNA gene OTU1, -2, or -3 grouping (Table 2). This was done to determine if there were any significant differences in the sizes of cells belonging to the different OTUs. The average width and length of cells belonging to OTU3 were statistically greater than those of OTU1 and -2 cells (Table 2). Cells belonging to OTU1 and OTU2, however, were not distinguishable from each other morphologically.

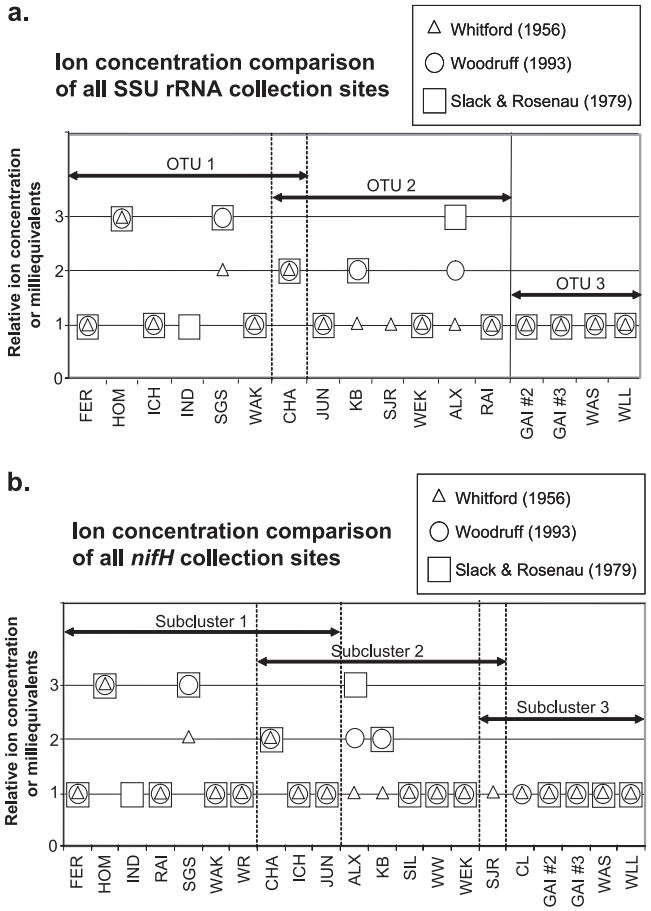

FIG. 3. Relative ion concentrations, from lowest to highest (1 to 3) segregated according to their corresponding phylogenetic grouping (Fig. 1), at all of the *Lyngbya* sp. collection sites included in this study. Table 3 contains descriptions of the *y*-axis groupings. (a) OTU1 to -3 are delineations from Fig. 1 based on a phylogenetic analysis of partial *L. wollei* SSU rRNA gene sequences. (b) S1 to -3 are delineations from Fig. 1 based on a phylogenetic analysis of partial *L. wollei nifH* gene sequences. For definitions of abbreviations, see the legend to Fig. 1.

When the cell length and width data were sorted on the basis of the specific SSU rRNA gene/*nifH* subcluster obtained from the same sample, it was apparent that cells belonging to the OTU3/*nifH* S3 subcluster in the phylogenetic analysis were significantly larger than cells associated with the other two subclusters. Cells belonging to the OTU1/S1 and OTU2/S2 subclusters, in turn, were similar in size and not distinguishable morphologically, despite being genetically distinct.

Environmental parameters. Each collection site from which *Lyngbya* samples were obtained was placed in a category based on major ion concentrations as defined by Whitford (25), Woodruff (26), and Slack and Rosenau (18) (Table 3). The goal was to determine if the hardness of the water correlated with specific genetically defined clusters. Collection sites where OTU3/S3 sequences were recovered generally had lower ion concentrations. In contrast, the OTU1/S1 and OTU2/S2 subclusters were more often found in environments exhibiting moderate-to-high ionic concentrations (Fig. 3). None of the OTUs were consistently correlated with average ambient dissolved inorganic N or phosphate concentrations measured at the site or with a specific range of N/P ratios (Fig. 4). Florida springs that were not sampled were not included in this nutrient comparison.

TABLE 4. Single-filament extraction sequences and their OTUs or clusters from the SSU rRNA gene and *nifH* phylogenetic analyses, respectively[a]

Collection site	SSU rRNA gene OTU	*nifH* subcluster(s)
Indian Springs	OUT1	S1
Wakulla Spring	OTU1	S1
Juniper Springs	NA[b]	S1, S2
Wekiwa Springs	OTU2	S2
Gainer Springs	OTU3	S3
Washington Spring	OTU3	S3
Williford Spring	OTU3	S3

[a] Fig. 1.
[b] NA, not applicable.

Geographical relationships. Within the limitations of our sampling, most of the collection sites tended to contain single OTUs. This was also true for the locations sampled multiple times (Fig. 1). There were no examples where OTU1, -2, and -3 all occurred at the same location. When the occurrence of OTUs was plotted geographically, there were no consistent geographical patterns apparent (Fig. 5). Within Florida, OTU3/S3 did come primarily from Williford, Washington, and Gainer Springs, which are located within close proximity to each other in the panhandle region. However, the same OTU was also recovered from the St. Johns River, FL, near Palatka, and City Lake in High Point, NC.

DISCUSSION

The combined phylogenetic analyses of the SSU rRNA and *nifH* genes, in conjunction with the morphological analysis, indicate that the *L. wollei* commonly identified in the literature represents two or possibly more species (19). Significant differences in filament size support two species, whereas the phylogenetic analyses of the SSU rRNA gene and *nifH* sequences obtained from the field samples indicate the existence of three

TABLE 3. *y*-axis values for Fig. 3 based on previous spring categorization studies involving dominant ion concentrations and milliequivalents

Rank	Ion concn	Whitford[a]	Woodruff[b]	Milliequivalents	Slack and Rosenau[c]
3	Highest	Mixed	Salt	Highest	Sodium chloride
2	High	Oligohaline	Mesohaline	High	Mixed
1	Low	Calcium bicarbonate	Calcium bicarbonate	Low	Calcium-magnesium bicarbonate

[a] Reference 25.
[b] Reference 26.
[c] Reference 18.

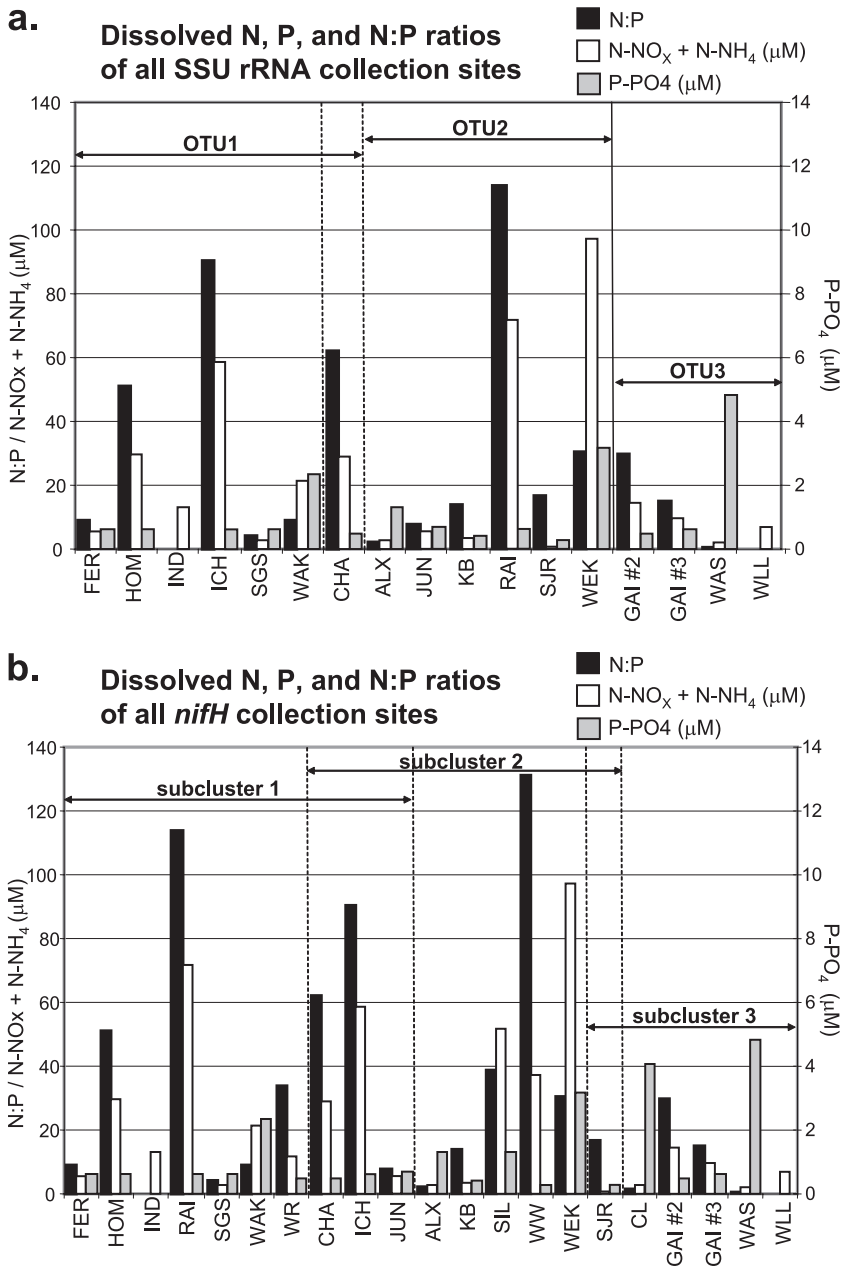

FIG. 4. N/P ratios and dissolved in organic N and P values for collection sites in this study. (a) OTU1 to -3 are delineations from Fig. 1 based on a phylogenetic analysis of partial *L. wollei* SSU rRNA gene sequences. (b) S1 to -3 are delineations from Fig. 1 based on a phylogenetic analysis of partial *L. wollei nifH* gene sequences. For definitions of abbreviations, see the legend to Fig. 1.

distinct species. The latter possibility is supported by the fact that the sequences in each of the three SSU rRNA gene OTUs showed >97% homology (Fig. 1). The argument for these being distinct species is further supported by the coherence between the specific SSU rRNA gene and *nifH* sequences obtained from any given mat sample or single filament (Fig. 1 and Table 4). The data indicated that the SSU rRNA gene cluster OTU1 obtained from a sample almost invariably corresponded to the recovery of the *nifH* S1 sequence from the same sample. The same was true for OTU2 with S2 and OTU3 with S3. It would be expected that if *L. wollei* were a single genetic entity not undergoing extensive genetic exchange with other OTUs, the same SSU rRNA gene and *nifH* sequences would be recovered from each putative *L. wollei* clump or filament analyzed. The phylogenetic data further showed that the *Lyngbya* SSU rRNA gene OTUs from this study were different from those in current shared databases including *Lyngbya majuscula* (Fig. 2). This is likely due to sampling bias rather than a regional distribution of these particular OTUs.

As stated above, the morphological data were only consistent with the existence of two species. Specifically, the measured dimensions of both OTU1/S1 and OTU2/S2 cells fell within the 24- to 65-μm width and 2- to 12-μm length ranges used to morphologically define *L. wollei* (19). These data in-

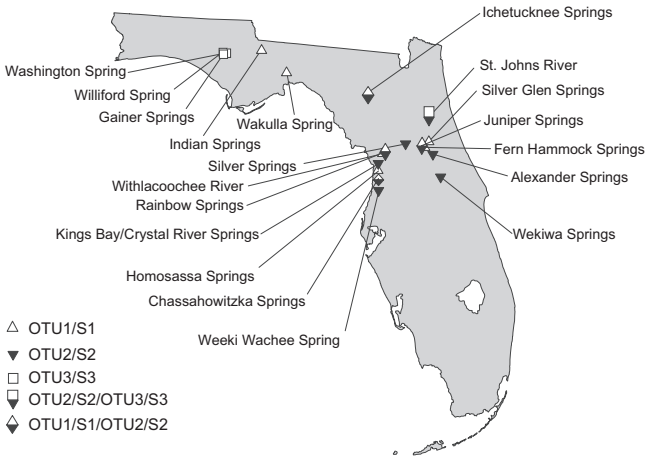

FIG. 5. Spring and river sites within Florida. Each collection site is represented by a symbol that corresponds to an OTU and S designation. The OTUs represent OTUs within the *Lyngbya* SSU rRNA gene library that we established in this study, and subclusters within a phylogenetic comparison of *Lyngbya nifH* sequences are represented by S1, -2, or -3 (Fig. 1).

dicate that OTU1/S1 and OTU2/S2 represent what is classically defined as *L. wollei* (19) and that this morphologically defined taxon actually contains two cryptic species or subspecies. However, the cells of OTU3/S3 were, on average, significantly wider (56.5 μm versus ~41 μm) and longer (7.4 μm versus ~5.5 μm) than those of OTU1/S1 and OTU2/S2 (Table 2). This size difference, in combination with distinct genetic differences, supports the conclusion that this is a distinct species.

Preliminary evidence also suggests that the putative species represented by OTU3 prefers habitats with a lower average ion content than the other species or subspecies. OTU1 and OTU2, in contrast, were found to occur in environments exhibiting a wide range of ion concentrations and milliequivalents (Fig. 3). These survey data indicated that neither OTU1 nor OTU2 showed any preference for an environment with a particular ion concentration, and this result was consistent with their being more broadly distributed geographically (Fig. 5). Interestingly, none of the OTUs were correlated with the overall availability of residual dissolved inorganic N or P or with differences in average N/P ratios (Fig. 4). The nutrient data collected and compared were from sites that contained *Lyngbya* mats. The selection of nutrient collection sites was therefore not random. The lack of any consistent N concentration for each OTU is likely due to the fact that *L. wollei* has the ability to rapidly and efficiently acquire dissolved forms of inorganic N (6) or to fix N when ambient N concentrations are low (12). Extensive nutrient studies of major springs in northern Florida have failed to find a correlation between the occurrence of *Lyngbya* and either dissolved inorganic N or P concentrations (23). This suggests that, from a management standpoint, while inputs of limiting nutrients might control overall biomass, they do not appear to be the primary factor affecting the distribution of these species.

Since these species are not found in all of the Florida springs that have experienced nutrient enrichment, there may be other limiting factors besides nutrients, including light, grazing, and temperature. Many springs are in protected national or state forests, and there is therefore a limited amount of light that can penetrate the tree canopy to reach the spring surface. *L. wollei* photosynthesizes the most at low light levels (~31 microeinsteins m^{-2} s^{-1}) below the mat's surface (21). Springs that are exposed to more light due to little or no tree canopy might support only limited or no *Lyngbya* growth because of photoinhibition. However, in this study we did not quantify tree canopy coverage nor did we find a previously existing record. With regard to the effects of grazing, a recent study suggests that the sheath that encases *L. wollei* cells is unpalatable and reduces herbivory (1). Therefore, grazing may not play a significant role in the limitation of *L. wollei* biomass. Temperature is also unlikely to be involved, as almost all Florida springs are consistently about 22°C.

Given the fact that OTU1 and -2 are widely distributed and do not seem to be limited by nutrients or the suite of environmental conditions associated with different average ionic concentrations or strengths, it is curious that these species or subspecies seldom occupy the same environment (Fig. 1). This raises the question of what may be responsible for this apparent broad but largely nonoverlapping distribution pattern. A possible answer is that the observed pattern is due to limited sampling in each given environment. However, for multiple samples that were taken at different places in a given environment, the same trend holds true. A wide geographic distribution limited in environmental diversity suggests that *Lyngbya* species have been randomly introduced into different environments. If this is so, it is possible that humans (or birds) may be accelerating the dispersal of filaments, as many of the environments where *Lyngbya* now occurs are used year-round by recreational swimmers and boaters. If these random *Lyngbya* introductions find suitable substrates on which to grow, then the final amount of biomass, and concomitant environmental degradation and toxin production, will depend on overall nutrient availability and light levels. Furthermore, if humans are the primary vector for *Lyngbya* distribution, then normal recreational activities are likely to continue spreading these species in an uncontrollable manner. The data also indicate that multiple species are involved and that at least two of them, commonly referred to as *L. wollei*, appear to have potentially different habitat preferences.

This study is one of the first to compare a large set of gene sequences (64 SSU rRNA gene sequences, 142 *nifH* sequences) from a single defined species from a large number of sites (21 freshwater sites). The original purpose of this study was to establish a set of SSU rRNA gene and *nifH* sequences that could be used to identify *L. wollei* accurately and relatively quickly. However, it has become apparent from our sequence analyses that the current definition of *L. wollei* includes a complex assemblage of species. The potential ramifications of this finding extend beyond taxonomy. For example, toxin production and nutrient management studies can now be targeted for each species. In terms of developing an effective management strategy for waters impacted by *L. wollei*, future work should be directed toward determining if the nutrient physiologies of these apparently distinct species are significantly different and, if so, whether specific phosphate input reductions or manipulation of the light environment would be required to control their abundance in differing ionic environments.

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